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**(54) 3'untranslated regions of alpha-amylase genes**

(57) An expression vector comprising a promoter capable of directing expression of a coding sequence in an angiosperm cell, and a 3' untranslated region of a rice  $\alpha$ -amylase gene, wherein, after the coding sequence is inserted into the vector, the 3' untranslated region and the coding sequence are transcribed into a single mRNA. Also featured are methods of producing a polypeptide in angiosperm cells using such a vector.

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**Description**Field of the Invention

5 [0001] This invention relates to the field of gene expression regulation in plant cells.

Background of the Invention

10 [0002] Sugar repression of gene expression is a ubiquitous regulatory mechanism for both prokaryotic and eukaryotic cells to adjust to changes in nutrient availability. In multicellular plants, expression of enzymes involved in carbohydrate metabolism is often feedback-regulated by sugar metabolites (Sheen, J., *Photosynth. Res.*, 39:427-438, 1994). For instance, carbohydrate depletion has been shown to enhance expression of a wide variety of genes involved in photosynthesis, reserve mobilization, and export processes (Koch, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 47:509-540, 1996).

15 [0003]  $\alpha$ -amylases are the major amylolytic enzymes for hydrolysis of starch stored in the endosperm during germination of cereal grains. These enzymes are encoded by a group of genes whose expression is repressed by sugars. In the embryos of germinating rice seeds, the transient expression of certain  $\alpha$ -amylase genes is thought to be induced by sugar depletion in the embryos following imbibition of the seeds, and suppressed by sugar influx from the endosperm as germination proceeds (Yu et al., *Plant Mol. Biol.* 30:1277-1289, 1996). Similarly, expression of  $\alpha$ -amylase genes in  
20 cultured rice suspension cells has also been shown to be suppressed by sugar present in the medium and induced in its absence (Yu et al., *J. Biol. Chem.*, 266:21131-21137, 1991, *Gene* 122:247-253 1992; Chen et al., *Plant J.*, 6:625-636, 1994). Further studies reveal that the transcription rate of  $\alpha$ -amylase genes in rice suspension cells increase in response to sucrose depletion in the culture medium (Sheu et al., *Plant J.*, 5:655-664, 1994). Protein expression systems have been established to make use of the sugar-responsive promoters of these  $\alpha$ -Amylase genes (US Patent No.  
25 5,460,952 to Yu et al.).

[0004] Sugar-regulated stability of rice  $\alpha$ -amylase mRNAs, on the other hand, has, also been investigated. Under transcription-inhibiting conditions, the mRNA half-lives of three  $\alpha$ -amylase genes were found to be prolonged by sucrose-starvation (Sheu et al., *J. Biol. Chem.*, 271:26998-27004, 1996). This result suggests that, in addition to enhanced transcription rate, increased mRNA stability may also contribute to the elevation of the steady-state levels of  
30 certain  $\alpha$ -amylase mRNA species in sucrose-starved cells. It is unclear, however, what mechanism is responsible for the increased degradation of  $\alpha$ -amylase mRNAs in cells provided with sucrose as compared to cells deprived of sucrose. It has been postulated that mRNA transcripts are selected for degradation in a sequence-dependent manner (Sachs, *Cell*, 74:413-421, 1993). Yet it remains largely unknown what sequences in RNA transcripts are responsible for targeting degradation in plants.

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Summary of the Invention

[0005] The invention is based on the discovery that the 3' untranslated regions of a rice  $\alpha$ -amylase gene can *cis*-regulate mRNA stability in angiosperm cells in a sugar-responsive manner.

40 [0006] Accordingly, the invention features expression vectors that comprise (i) a promoter capable of directing expression of a coding sequence in an angiosperm cell; and (ii) a 3' untranslated region (3'UTR) of a rice  $\alpha$ -amylase gene (i.e., a sequence that is identical to a given 3'UTR of a rice  $\alpha$ -amylase gene, or a functional variant of the given 3'UTR). In these vectors, the 3'UTR is no longer linked to an  $\alpha$ -amylase coding sequence as in its cognate  $\alpha$ -amylase gene or a cDNA obtained therefrom. After a coding sequence (e.g., a non- $\alpha$ -amylase coding sequence) is inserted into the vec-  
45 tors, the coding sequence and the 3'UTR are transcribed into a single ribonucleic acid, e.g., messenger ribonucleic acid (mRNA). An angiosperm cell is a monocotyledon, such as cereal (e.g., rice, barley, and wheat), or dicotyledon cell.

[0007] The promoter in the expression vectors can be a ubiquitously active one, such as one that is derived from an actin gene, cauliflower mosaic virus 35S ("CaMV35S") RNA or a ubiquitin gene, or an inducible one, such as one that is derived from an  $\alpha$ -amylase (e.g., the rice  $\alpha$ Amy3,  $\alpha$ Amy6,  $\alpha$ Amy7,  $\alpha$ Amy8, or  $\alpha$ Amy10 gene, which are also sugar-  
50 responsive, see U.S. Patent No. 5,460,952), invertase, sucrose synthase, patatin,  $\beta$ -amylase, or sporamin gene. Other appropriate transcription regulatory elements such as enhancers can also be included in the vectors.

[0008] If the  $\alpha$ -amylase 3'UTR is used to stabilize mRNA in the absence or low concentration of sugars, the sugar-suppressible promoters, such as the photosynthetic gene and  $\alpha$ -amylase gene promoters can be used.

55 [0009] The coding sequence can encode, for example, a polypeptide, or an antisense RNA that interferes with the function of another RNA (e.g., mRNA, transfer RNA, ribosomal RNA, and small nucleolar RNA).

[0010] The 3' untranslated region is typically selected for its ability to *cis*-regulate RNA stability in a sugar-responsive manner. The 3' untranslated region can be derived from, for example, a 3' untranslated region (3'UTR) of a rice  $\alpha$ -amylase 3 ( $\alpha$ Amy3) gene, i.e., an  $\alpha$ Amy3 gene of any rice strain; that is, the 3'UTR of the vectors can be identical to, or a

functional variant (e.g., containing one or more silent mutations) of, a rice  $\alpha$ Amy3 3'UTR. The 3'UTR can be obtained either by chemical synthesis or recombinant technology. The 3'UTRs include, but are not limited to, sequences consisting of 20 or more consecutive nucleotides from SEQ ID NO:1, e.g., sequences spanning nucleotides 44-320, 44-75, 75-320, 75-124, 124-320, 124-154, 154-320, 154-184, 184-320, 184-237, 237-320, 237-258, 258-320, 3-135, 119-199, and 182-276 of SEQ ID NO:1. The 3'UTR can be in either correct or reverse orientation in relation to the promoter's direction.

**[0011]** The expression vectors can further comprise a signal-peptide-encoding sequence (e.g., one that is derived from a rice  $\alpha$ -amylase gene) downstream of the promoter, so that the polypeptide products expressed from the expression vectors can be secreted by host cells.

**[0012]** To facilitate cloning, the expression vectors of the invention may comprise multiple restriction sites downstream of the promoter so that a coding sequence can be conveniently inserted into the vector between the promoter and the rice 3'UTR.

**[0013]** The invention also features methods of producing a polypeptide in angiosperm cells. These methods utilize an expression construct containing a coding sequence for a polypeptide of interest, linked operably to a promoter capable of directing its expression in an angiosperm cell, and a 3' untranslated region of a rice  $\alpha$ -amylase gene. The coding sequence and the untranslated region are not naturally transcribed into a single mRNA, but are so in the construct. Such a construct is also within the scope of the invention.

**[0014]** In a method of the invention, appropriate angiosperm host cells are first transformed by the expression construct, and the transformed cells (including cells derived therefrom, e.g., through replication, regeneration, or differentiation) are then subjected to a sugar-free, or sugar-containing, environment to promote expression of the polypeptide. If the 3'UTR contained in the expression construct enhances mRNA stability when the host cells are placed in a medium or environment containing sugar (at, e.g., 1 mM to 300 mM), then the transformants are subjected to such a medium or environment to promote expression of the polypeptide; examples of such a 3'UTR include, but are not limited to, sequences spanning nucleotides 119-199, 124-320, 154-320, and 258-320 of SEQ ID NO:1. On the other hand, if the 3'UTR enhances mRNA stability when the host cells are placed in a sugar-free environment, the transformants can be placed in such an environment to promote the polypeptide's production; examples of such a 3'UTR include, but are not limited to SEQ ID NO:1, sequences spanning nucleotides 3-135, 44-320, 75-320, 184-320, 237-320, and 182-276 of SEQ ID NO:1. A sugar can be sucrose, glucose, fructose, maltose, or any polysaccharide as long as it is metabolizable and provides a carbon source for cells.

**[0015]** A sugar-free (i.e., sugar-starved) environment refers to an environment, e.g., a culture medium, that does not contain any sugar, or an environment whose sugar concentration is so low that it does not support the proliferation or respiration of cells. Occurrence of sugar starvation depends on multiple factors such as sugar concentration, the cell-to-medium volume ratio, and the length of incubation time in a medium.

**[0016]** Of course, the above transformants can also be regenerated to tissue or even a whole plant (i.e., a transgenic plant) to promote expression of the polypeptide. Seeds of such a transgenic plant may also be germinated to promote expression of the recombinant polypeptide.

**[0017]** To further promote expression of the polypeptide, the promoter in the expression construct can be sugar-responsive in synergy with the 3'UTR. For instance, if the 3'UTR enhances mRNA stability in the absence of sugar, then a promoter that is activated by sugar starvation (e.g., a promoter derived from a rice  $\alpha$ Amy3,  $\alpha$ Amy6,  $\alpha$ Amy7,  $\alpha$ Amy8, and  $\alpha$ Amy10 gene) can be employed in the expression construct. By way of example, a coding sequence in association with an  $\alpha$ Amy3 promoter and the full length  $\alpha$ Amy3 3'UTR is highly expressed in sugar-starved cells.

**[0018]** To facilitate recovery of the expressed polypeptide, a signal-peptide-encoding sequence can be integrated into the expression construct at an appropriate position, e.g., between the promoter and a coding sequence for the polypeptide. As a result, the polypeptide can be secreted to the culture medium, and thereby conveniently recovered.

**[0019]** Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

#### Brief Description of the Drawings

**[0020]**

Fig. 1 is a set of schematic diagrams showing the various plasmid constructs used for rice transformation. Positions of primers (arrowheads; for sequences, see Table 1) for CRT-PCR and expected lengths of PCR products are also shown. All the constructs contain the maize ubiquitin (*Ubi*) promoter, the *Ubi* first intron (*Ubi*-I), the luciferase (*Luc*) or  $\beta$ -glucuronidase (*Gus*) coding sequence, and a 3' UTR of  $\alpha$ Amy3, *Agrobacterium* nopaline synthase (*Nos*), or actin 1 (*Act1*) gene. Fragments (or subdomains) I, II, and III derived from the various regions of the full-length  $\alpha$ Amy3 3'UTR were inserted into pLN between the *Luc* coding region and the *Nos* 3'UTR. The nucleotide sequences are numbered from the first nucleotide after the translation stop codon TGA.

Fig. 2 is a graph showing that the full-length  $\alpha$ Amy3 3'UTR and three fragments thereof mediate sugar-dependent decrease or increase of heterologous mRNA accumulation in transgenic rice suspension cells.

Fig. 3 is a set of graphs showing that various  $\alpha$ Amy3 3' UTR fragments affect mRNA half-life in a sugar-responsive manner. The time on the horizontal axis is shown in hours. The open and filled circles indicate mRNA from cells grown in sugar-plus (+S) and sugar-minus (-S) medium, respectively. The dashed line indicates the time at which 50% of mRNA remained.

Fig. 4 is a set of schematic diagrams showing the structures of chimeric genes containing various 5' deletions of the full-length  $\alpha$ Amy3 3'UTR. The nucleotide sequences are numbered from the first nucleotide after the translation stop codon TGA. Arrowheads indicate the positions of primers used for CRT-PCR. Expected sizes of the gene-specific DNA fragments generated by CRT-PCR are indicated in bp (base pairs).

#### Detailed Description

[0021] Described below is the discovery that the full-length 3' untranslated region (SEQ ID NO:1) of a rice  $\alpha$ -amylase gene ( $\alpha$ Amy3) enhances mRNA stability in the absence of sugar (e.g., sucrose and glucose), and promotes mRNA degradation in the presence of sugar; and that fragments of the full-length  $\alpha$ Amy3 3' untranslated region (3'UTR) can independently regulate mRNA accumulation in a sugar-responsive manner.

[0022] The expression vectors of the invention, which contain a 3'UTR of a rice  $\alpha$ -amylase gene, can be generated by standard recombinant technology (see, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, New York, 1989; and Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York, New York, 1993). For instance, a DNA fragment containing the 3'UTR is first obtained by polymerase chain reaction, using a rice  $\alpha$ -amylase cDNA as a template, and then inserted into a plasmid that has already had an appropriate angiosperm promoter (or additionally, a coding sequence downstream of the promoter) in place.

[0023] Plant cells can be transfected with the expression vectors using standard methods, such as electroporation, particle bombardment, microinjection, ultrasonic method, polyethylene glycol-mediated protoplast transformation, poly-L ornithine method, calcium phosphate method, and Agrobacterium-mediated transformation system (see, e.g., U.S. Patent No. 5,460,952, columns 17-18; and references the patent).

[0024] Plant cells transformed by expression constructs (i.e., expression vectors containing a sequence encoding, e.g., a desired polypeptide) can be maintained and expanded in a sugar-free or sugar-containing culture medium to promote expression of the polypeptide. The polypeptide can be subsequently extracted and purified from the cells using standard techniques. Alternatively, the polypeptide can be isolated from the culture medium if the expression constructs contain a signal peptide-encoding sequence so that the polypeptide is secreted into the medium (Chen et al., Plant J., 6:625-638, 1994; Sijmons et al., Bio/Technology, 8:217-221, 1990). One advantage of purifying proteins directly from a culture medium is that the amount of contaminant protein is substantially lower in culture media than in cell extracts.

[0025] Transformed plant cells can also be regenerated to transgenic tissue or a transgenic plant by well known techniques (see, e.g., Datta et al., Bio/Technology, 8:736-740, 1990; Peng et al., Plant Cell Reports, 9:168-172, 1990; Yang et al., Plant Cell Reports, 7:421-425, 1988; U.S. Patent Applications 08/509,962 and 08/639,792). The recombinant polypeptide can then be recovered from the transgenic tissue or plant (or a part of the plant, such as its leaves, sheaths, stems, seeds, and roots, depending on the tissue-specificity of the promoter used). Both photosynthetic leaves and the endosperm of germinating seeds provide high amounts of sugar; thus, recombinant polypeptides can be harvested from these tissues if the polypeptide expression vectors contain 3'UTRs that stabilize mRNAs in a sugar-containing environment.

[0026] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, exemplary methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[0027] The following data and protocols are used as examples to illustrate, but not limit, the expression vectors, constructs, and methods of the invention.

#### Example 1

[0028] This example concerns the sugar-regulated stability of an  $\alpha$ Amy3 mRNA. Studies disclosed in this example demonstrate that the full-length  $\alpha$ Amy3 3'UTR and two of its three subdomains, i.e., subdomains I and III, function as destabilizing determinants in mRNA turnover in the presence of sugars.

## Plant Materials

[0029] The rice variety used for transformation was *Oryza sativa* L. cv. Tainan 5. Embryogenic calli were initiated as described by (Chan et al. *Plant Mol. Biol.*, 22:491-506, 1993). except that the medium was replaced with MS medium (Murashige, *Physiol. Plant*, 15:473-497, 1962). containing 1% agarose (w/v), 3% sucrose, and 2 µg/ml 2,4-Dichlorophenoxyacetic acid ("2,4-D"; pH 5.8). After four weeks, suspension cultures were initiated by transferring the calli into a liquid MS medium containing 3% sucrose, 2 µg/ml 2,4-D, 0.2 µg/ml kinetin, and 0.1 µg/ml GA<sub>3</sub>. The suspension culture was shaken on a reciprocal shaker at 120 rpm and incubated at 26°C in the dark. Suspension cells were subcultured weekly prior to bombardment.

## Transformation of rice suspension cells

[0030] In a volume of 70 µl, 1.5 mg of gold particles (Bio-Rad) with an average size of 1 µm in diameter were coated with 2 µg of test plasmid and 1 µg of pTRA151 (a plasmid containing hygromycin resistant gene (Zheng, et al., *Plant Physiol.*, 97:832-835, 1991) by a CaCl<sub>2</sub>/spermidine precipitation method (Cao et al., *Plant Gene Transfer*, eds. Lamb, C.J. & Beachy, R.N., Wiley-Liss, New York, pp. 21-33, 1990). Rice suspension cells were bombarded with the coated gold particles as described by Cao et al. (*Plant Cell Rep.*, 11:586-591., 1992) using a helium gas retrofitted BIONIS-TIC™PDS-1000 Particle Delivery System (Bio-Rad). Bombarded cells were transferred to fresh MS solid medium and incubated at 26°C in the dark for 6 days before being transferred to MS solid medium containing 50 µg/ml hygromycin. After 4 weeks, all putative transformed calli were subcultured to MS liquid medium containing 50 µg/ml hygromycin to generate suspension cell culture.

## Determination of mRNA half life

[0031] Transgenic cells were cultured in suspension in sucrose-containing (+S) medium for 96 h and transferred to sucrose-free (-S) medium for 24 h. Actinomycin D (ActD) was then added to the medium to a final concentration of 10 µg/ml. Cells were incubated for another 12 hours (h) and then divided into two halves. One half of the cells were transferred to a +S medium containing ActD, while the other half of cells were transferred to a -S medium containing ActD. Cells were harvested after 0.5 to 9 h, and total cellular RNAs were extracted, electrophoresed on polyacrylamide gel, and transferred to a nylon membrane. Northern blot analysis of the RNAs was performed using radioactive probe that hybridized to the luciferase gene (*Luc*) sequence. Radioactive signals were quantified using a PhosphorImager. The mRNA level in each lane was normalized to the rRNA level (obtained using an rRNA probe). The logarithm values of mRNA levels were subjected to linear regression analysis and plotted as a function of time.

## Results

### (1) The Full-Length $\alpha$ Amy3 3'UTR and Certain Subdomains Thereof Mediate Sugar-Dependent Repression of mRNA Accumulation

[0032] Effects of an  $\alpha$ Amy3 3'UTR on the accumulation of a heterologous mRNA were investigated in transgenic rice cell lines. Used for transformation were plasmids pLAm and pLN (see Example 2, *infra*), which contained (i) the full-length  $\alpha$ Amy3 3'UTR, and (ii) the *Agrobacterium* nopaline synthase gene (*Nos*) 3'UTR, respectively, downstream from the ubiquitin (*Ubi*) promoter and the *Luc* coding region. Also used were plasmids pLIN, pLIIN, and pLIIN (see Example 2, *infra*), which respectively contained fragments I, II, and III of the full-length  $\alpha$ Amy3 3'UTR between the *Luc* coding region and the *Nos* 3'UTR. Schematic structures of these plasmids are shown in Fig. 1. Identification of transgenic rice calli was confirmed by Southern analysis of genomic DNA.

[0033] Five independently-transformed cell lines were randomly selected for each plasmid construct, and levels of the *Luc* mRNA were examined by RNA dot blot analysis using a segment derived from the *Luc* coding sequence as a probe (Fig. 2). Radioactive signals generated by the hybridized *Luc* mRNA in each dot were quantified using a PhosphorImager (Molecular Dynamics). The ratio of mRNA levels in the absence (-S) *versus* presence (+S) of sucrose was determined for each transformed cell line. Logarithm value of each ratio (open circle) was then used to plot the graph. Bold horizontal bars represent the average value for each construct. A ratio higher than log<sub>10</sub>° (i.e., 1) indicates that the *Luc* mRNA level was increased by sucrose starvation, whereas a ratio lower than log<sub>10</sub>° indicates the *Luc* mRNA level was decreased by sucrose starvation.

[0034] Fig. 2 shows that the *Luc* mRNA levels in cell lines transformed with pLAm, pLIN, and pLIIN were increased by sucrose starvation; in contrast, the *Luc* mRNA levels in cell lines transformed with pLN and pLIIN were decreased by sucrose starvation. In all the cell lines tested, levels of the endogenous  $\alpha$ Amy3 mRNA were increased, while levels of the endogenous actin (*Act*) mRNA were decreased, by sucrose starvation.

[0035] The above results were consistent with those of the transient assays in rice protoplasts (Example 2, *infra*): The full-length  $\alpha$ Amy3 3'UTR and at least two of its different subdomains specifically mediate sugar-dependent decrease of the accumulation of the *Luc-Nos* mRNA (i.e., mRNA containing the *Luc* coding sequence and the *Nos* 3'UTR) whose accumulation otherwise is increased by sugar provision. Of note, in the presence of sucrose, levels of the *Luc* mRNA in cells transformed with pLAm, pLIN, and pLIIN were still detectable, while those of the endogenous  $\alpha$ Amy3 mRNA were not. This was probably due to a higher transcriptional activity of the *Ubi* promoter than the  $\alpha$ Amy3 promoter in sucrose-provided cells.

## (2) The Full-Length $\alpha$ Amy3 3'UTR Functions as a Sugar-Responsive mRNA Stability Determinant

[0036] To determine whether the low accumulation of the  $\alpha$ Amy3 3'UTR-containing chimeric mRNAs in sucrose-provided cells was due to their rapid turnover, the half-lives of the chimeric mRNAs were measured by shutting off transcription of the pLAm-transformed cells. Half-lives of mRNAs were determined by Northern analysis following transcription inhibition by ActD (Sheu, et al., *Plant J.*, 5:655-664, 1994).

[0037] Levels of the *Luc- $\alpha$ Amy3* (i.e., mRNAs containing the *Luc* coding sequence and an  $\alpha$ Amy3 3'UTR) and endogenous  $\alpha$ Amy3 mRNAs were low or undetectable in cells provided with sucrose, but increased significantly after cells were sucrose-starved for 24, 36, or 45 h. The *Luc- $\alpha$ Amy3* and  $\alpha$ Amy3 mRNAs that accumulated after cells had been starved for 24 h and then treated with ActD for 12 h decayed slowly in cells subsequently starved for sucrose, but decayed rapidly in cells subsequently provided with sucrose. In contrast, the levels of actin mRNA were high in cells provided with sucrose, but were low in cells starved for sucrose or provided with ActD.

[0038] The relative half-lives of *Luc- $\alpha$ Amy3* and  $\alpha$ Amy3 mRNAs were both found to be 70 minute (min) in cells provided with sucrose. The half-lives increased to 300 and 320 min, respectively, when the cells were starved for sucrose. These results demonstrate that the *Luc* mRNA carrying the full-length  $\alpha$ Amy3 3'UTR is more stable in cells starved for sucrose.

## (3) Various Subdomains of the Full-Length $\alpha$ Amy3 3'UTR Act Independently as Sugar-Responsive mRNA Stability Determinants

[0039] Since insertion of subdomains I or III of the full-length  $\alpha$ Amy3 3'UTR between the *Luc* coding region and the *Nos* 3'UTR conferred sugar-dependent decrease of mRNA accumulation (Fig. 2), we sought to determine whether the two subdomains confer differential stability to the heterologous mRNA. The turnover of mRNAs in transgenic rice suspension cells was determined by Northern analysis following transcription inhibition by ActD. In the pLIN- and pLIIN-transformed cells, the *Luc-Nos* and *Luc-II-Nos* (i.e., mRNA containing the *Luc* coding sequence, the subdomain II of the  $\alpha$ Amy3 3'UTR, and the *Nos* 3'UTR) mRNAs decayed slowly in the presence of sucrose, but decayed rapidly in the absence of sucrose. In contrast, in the pLIN- and pLIIN-transformed cells, the *Luc-I-Nos*, *Luc-III-Nos* mRNAs as well as the endogenous  $\alpha$ Amy3 mRNA decayed rapidly in the presence of sucrose, but decayed slowly in the absence of sucrose. These results demonstrate that subdomains I and III, but not subdomain II, increase the stability of the *Luc-Nos* mRNA in response to sucrose starvation.

[0040] Half-lives of the above *Luc* mRNAs were determined and shown in Fig. 3. In the absence of sucrose, the half-life of the *Luc-Nos* mRNA was 55 min, which was similar to that of the *Luc-I-Nos* (60 min) and *Luc-II-Nos* (55 min) mRNA but 3.4-fold less than that of the *Luc-III-Nos* (185 min). In the presence of sucrose, the half-life of *Luc-Nos* mRNA was 200 min, which was 6.7-fold of the *Luc-I-Nos* mRNA (30 min), 2-fold of the *Luc-II-Nos* mRNA (100 min), and 2.5-fold of the *Luc-III-Nos* mRNA (80 min). These results suggest that in the absence of sucrose, fragment III delays the decay of the *Luc-Nos* mRNA. However, in the presence of sucrose, all three fragments promote the decay of the *Luc-Nos* mRNA.

### Example 2

[0041] In this example, the effects of various  $\alpha$ Amy3 3'UTRs (i.e., various regions of the full-length  $\alpha$ Amy3 3'UTR) on mRNA accumulation in rice protoplasts were examined with a highly sensitive method termed co-amplification reverse transcriptase-polymerase chain reaction (CRT-PCR). Unlike Northern or dot blot analysis, CRT-PCR allows simultaneous detecting and/or quantitation of multiple RNA transcript species. In this example, by "the  $\alpha$ Amy3 3'UTR" is meant that the full length  $\alpha$ Amy3 3'UTR.

### Plant Materials and Protoplast Isolation

[0042] Suspension cell culture of rice (*Oryza sativa* cv. Tainan 5) were propagated as described previously (Yu et al., *J. Biol. Chem.* 266:21131-21137, 1991). Protoplasts were isolated from rice suspension cells according to the meth-

ods described by Lee et al. (1989). For transfection, protoplast concentration was adjusted to  $1 \times 10^7$  protoplasts/ml in KPR medium (Kao and Michayluk, 1975).

#### Plasmid Construction

**[0043]** Plasmid p $\alpha$ Amy3 was constructed using the pBluescript-KS(+) vector (Stratagene) and contains a 1.7-kb insert derived from a rice  $\alpha$ Amy3 cDNA (Sheu et al., *J. Biol. Chem.*, 271:26998-27004, 1996). Plasmid pcRAc1.3 was constructed using the pBluescriptII-KS vector (McElroy et al., *Plant Mol. Biol.*, 14:163-171, 1990) and contains a 1.4-kb insert derived from a rice actin cDNA (*Act1*). These two plasmids were used as templates for PCR amplification of 3'UTRs.

**[0044]** All of the plasmids contain a maize ubiquitin gene (*Ubi*) promoter, a luciferase (*Luc*) or  $\beta$ -glucuronidase (*Gus*) coding sequence linked to the *Ubi* promoter, and a 3'UTR of the  $\alpha$ Amy3, *Act1*, or *Nos* gene. To generate these plasmids, the PCR amplified 3'UTRs of  $\alpha$ Amy3 and *Act1* containing *Xba*I and *Sac*II sites at two ends were first subcloned into pBluescriptII-KS(+), resulting in plasmids pMTC37 and pMTC39, respectively. The *Hind*III-*Bam*HI DNA fragment containing the *Ubi* promoter, exon 1, intron 1, and part of exon 2 from pAHC18 (Bruce et al., *Proc. Natl. Acad. Sci.*, 86:9692-9696, 1989) was then inserted upstream of the  $\alpha$ Amy3 3'UTR in pMTC 37 to generate plasmid pMTC24. To generate pMTC241, pMTC24 was linearized with *Kpn*I and *Hind*III, blunt-ended, and re-ligated to remove the multiple cloning sites. The *Luc* coding sequence was then excised from pAHC18 by *Bam*HI and inserted into the same site between the *Ubi* promoter and the  $\alpha$ Amy3 3'UTR in pMTC241 to generate plasmid pLAm. The *Nos* 3'UTR was excised from pAHC18 by *Bam*HI and *Sac*I. Then the plasmids pLAc and pLN were similarly constructed as was pLAm, except that they contained the *Act1* and *Nos* 3'UTRs downstream of the *Luc* gene, respectively.

**[0045]** To construct the plasmids containing serial deletions from the 5' end of the full-length  $\alpha$ Amy3 3'UTR, pLAm was linearized at the *Kpn*I and *Spe*I sites located between the *Luc* coding sequence and the  $\alpha$ Amy3 3'UTR, and then subjected to Exonuclease III (Erase-a-Base kit, Promega) digestion at 15°C for 2 min. Samples were taken at 30-second intervals. The resulting plasmid DNAs were amplified by PCR, using LuRT2 and 3RT2 (Table 1) as primers. PCR products were examined by electrophoresis on 6% nondenaturing polyacrylamide gel; and plasmids yielding the desired lengths of products, i.e., containing the desired 5'-deleted  $\alpha$ Amy3 3'UTR, were selected. These plasmids were designated as pLAmD1 through pLAmD7 (Fig. 4). The exact deletion points were determined by DNA sequence analysis. The *Luc* coding sequence in pLAm, pLAmD2, pLAmD3, and pLAmD5 were replaced with *Gus*, generating plasmid pGAm, pGAmD2, pGAmD3, and pGAmD5.

Table 1: Oligonucleotides used in making 3' UTR constructs and in CRT-PCR analyses

Name of primer	Region in a gene	Position in a gene	Nucleotide Sequence (5' → 3')	
1-53	5' end of $\alpha$ Amy3 3'UTR	-3 - 15 <sup>b</sup>	TAA <u>TCTAGA</u> TAGCGGGCTCAAGCCCTA XbaI	(SEQ ID NO:2)
3RT2	3' end of $\alpha$ Amy3 3'UTR	289 - 303 <sup>b</sup>	TCC <u>CCGCGG</u> TCAGTGAGTTTAA SacII	(SEQ ID NO:3)
SP3	3' end of $\alpha$ Amy3 3'UTR	248 - 263 <sup>b</sup>	TATATAATGTCAGGTT	(SEQ ID NO:4)
A53	5' end of Act1 3' UTR	-3 - 12 <sup>b</sup>	TGC <u>TCTAGA</u> TAATTCTTCGACCCA XbaI	(SEQ ID NO:5)
A30	3' end of Act1 3'UTR	228 - 242 <sup>b</sup>	TCC <u>CCGCGG</u> TGGTACCCGCATCA SacII	(SEQ ID NO:6)
NosRT1	3' end of Nos 3'UTR	168 - 180 <sup>b</sup>	GCGGGACTCTSSTCATAAAA	(SEQ ID NO:7)
3RT1	Coding region of $\alpha$ Amy3	1096 - 115 <sup>a</sup>	ACGGCATCAACGCCGGGAGC	(SEQ ID NO:8)
ART1	Coding region of Act1	914 - 932 <sup>a</sup>	CTGATGGACAGGTTATCACC	(SEQ ID NO:9)
LuRT2	Coding region of Gus	1667 - 1686 <sup>a</sup>	TCAGCGATGACGAAATTCCT	(SEQ ID NO:10)
GRT1	$\alpha$ Amy3 3'UTR	1388 - 1407 <sup>a</sup>	TCTCTTTGATGTGCTGTGCC	(SEQ ID NO:11)
3R1	$\alpha$ Amy3 3'UTR	3 - 17 <sup>b</sup>	TGAGCTC GGCTCAAGCCCTAAA SacI	(SEQ ID NO:12)
3R2	$\alpha$ Amy3 3'UTR	121 - 135 <sup>b</sup>	TGAGCTC CTACAATCGGATACA SacI	(SEQ ID NO:13)
3R3	$\alpha$ Amy3 3'UTR	120 - 134 <sup>b</sup>	TGAGCTC TTGTATCCGATTGTA SacI	(SEQ ID NO:14)
3R4	$\alpha$ Amy3 3'UTR	185 - 199 <sup>b</sup>	TGAGCTC TGGCAAGCTACATAT SacI	(SEQ ID NO:15)
3R5	$\alpha$ Amy3 3'UTR	182 - 196 <sup>b</sup>	TGAGCTC GGCATATGTAGCTT SacI	(SEQ ID NO:16)
3R6	$\alpha$ Amy3 3'UTR	252 - 266 <sup>b</sup>	TGAGCTC CCTGAACCTGAACCT SacI	(SEQ ID NO:17)

a. The first nucleotide of translation initiation codon ATG at +1  
b. The first nucleotide after translation stop codon as +1.

[0046] To construct plasmids containing other  $\alpha$ Amy3 3'UTRs, DNA fragments encompassing the regions were first obtained by PCR, using pMTC37 as a template and pairs of oligonucleotides flanking these regions as primers (Table 1). Primers 3R1 and 3R2 were used to generate fragment I (i.e., subdomain I); primers 3R3 and 3R4 for fragment II (i.e., subdomain II); and primers 3R5 and 3R6 for fragment III (i.e., subdomain III). These fragments all contained, at both ends, a *SacI* site introduced by the PCR primers. The PCR products were hence digested with *SacI* and inserted into the same site between the *Luc* coding sequence and the *Nos* 3'UTR in pLN. Fragments I, II, and III were inserted into pLN in obverse orientation to generate pLIN, pLIIN, and pLIIN, and in reverse orientation to generate pLIRN, pLIIRN, and pLIIRN.

#### Polyethylene Glycol (PEG)-Mediated Transfection of Rice Protoplasts

[0047] The PEG-mediated transfection of rice protoplasts was performed by a method as described by Lyznik et al, (Biotechniques, 10:294-300, 1991), with modification. 100  $\mu$ l of rice protoplasts were transferred to a 1.5-ml Eppendorf tube and mixed with 5  $\mu$ g of supercoiled plasmid DNA. If cotransfection was performed, 4  $\mu$ g of test plasmid and 2  $\mu$ g of reference plasmid were mixed and added. Subsequently, 10  $\mu$ g of salmon sperm DNA, and 100  $\mu$ l of F-medium (140 mM NaCl, 5 mM KCl, 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM glucose, and 125 mM CaCl<sub>2</sub> · 2H<sub>2</sub>O, pH 7.0) containing 40% (w/v) PEG (mol. wt. 8000, Sigma) were also added to the plasmid/cell mix. After incubation at room temperature for 20 min, 0.2 ml of DNA/cell mix was added into a well of a 24-well microculture plate. The well contained 2 ml of solidified 0.2% low melting temperature agarose (Gibco-BRL) in modified KPR medium. After 18 h of incubation, the protoplasts were transferred into a 1.5 ml Eppendorf tube and centrifuged for 5 seconds. The protoplasts were resuspended in 0.5 ml of modified KPR medium containing glucose or mannitol, transferred into a well of another 24-well microculture plate, and incubated for various lengths of time.

#### Primers

[0048] Primers for the coding regions of the  $\alpha$ Amy3, Act1, *Luc*, or *Gus* gene, and for a 3'UTRs of  $\alpha$ Amy3, Act1, or



*Nos* gene were designed so that the molecular weight of the RT (reverse transcription)-PCR products for the various endogenous and transgene transcripts were different from each other and could be distinguished by agarose gel electrophoresis. Locations and nucleotide sequences of these primers are shown in Figs. 1 and 4, and listed in Table 1.

## 5 Reverse Transcription-Polymerase Chain Reaction

[0049] Total RNA was extracted from protoplasts by the method of Verwoerd et al. (1989). Twenty µg of total RNA was incubated in a volume of 20 µl with 5 µM DTT, 2 units/µl RNasin (Promega), and 1 unit of RNase-free DNase I (Promega). After 15 min of incubation at 37°C, the RNA was incubated at 80°C for 3 min and placed on ice. Reverse transcription of the RNA was performed in 20 µl of the reaction mix, which contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl<sub>2</sub>, 10 µg of the RNA, 50 pmole oligo (dT)<sub>18</sub> primer, 5 mM DTT, 0.5 mM each dNTP, 2 units/µl RNasin, and 10 units/µl Maloney murine leukaemia virus reverse transcriptase (MMLV-RTase). The reaction was carried out at room temperature for 10 min, then at 37°C for 1 h, and was terminated at 94°C for 5 min. The RT product mix was used as a cDNA stock and could be stored at -70°C for several months.

15 [0050] PCR was carried out in a volume of 50 µl containing 2.5 µl RT product mix prepared above, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 0.1 mM each dNTP, 100 pmole each primer, 10% DMSO, and 3 units super Taq DNA polymerase (Promega). Cycling was controlled by a programmable thermal cycler (Hybaid). Denaturation was at 94°C for 1 min; primer annealing at 50°C for 1.5 min; and primer extension at 72°C for 2 min. The reaction was run for 30 cycles before a final 10 min of primer extension at 72°C. The RT-PCR products were fractionated in 2% agarose gel  
20 and visualized by ethidium bromide staining.

## Primer Extension Analysis

25 [0051] Primer extension analysis was performed by a method essentially the same as described by (Sutliff et al., Plant Mol. Biol., 16:579-591, 1991), except that 10 µg of total RNA was used as a template and the primer extension products were analyzed on 1% agarose gel.

## Results

### 30 (1) CRT-PCR: A method of Measuring Steady-State mRNA Levels of Individual Several Genes Simultaneously

[0052] To define *cis*-acting sequences in the  $\alpha$ -amylase mRNA that control gene expression *in vivo*, we sought to develop a method for measuring mRNA levels in rice protoplasts. CRT-PCR was chosen for this purpose, because it can measure individual steady-state mRNA levels of several genes simultaneously. The principle of CRT-PCR is illustrated in Fig. 1: At least two sets of gene-specific primers are included in the same RT-PCR reaction to amplify cDNAs from mRNAs of interested genes. Positions of the primers corresponding to individual genes are designed so as to produce DNA fragments of distinct sizes that are diagnostic for each mRNA. The relative intensity of the DNA bands obtained after electrophoresis and ethidium bromide staining correlate with the relative amounts of different mRNAs present in the sample, thus allowing simultaneous quantification of transcripts from different genes.

40 [0053] The validity of CRT-PCR was demonstrated in a reconstruction experiment. In this experiment, various amounts of mRNA purified from rice suspension cells cultured with or without glucose for 2 days were reverse transcribed; and the RT products were PCR-amplified, using 2 pairs of primers specific for the cDNAs of the *Act1* and  $\alpha$ *Amy3* mRNAs (Fig. 1). Two PCR products were produced, one corresponding to the *Act1* cDNA (640 bp) and the other to the  $\alpha$ *Amy3* cDNA (500 bp). As shown in ethidium bromide-stained agarose gel, the band intensities of both  
45 products decreased with reduced mRNA input. The band intensity of  $\alpha$ *Amy3* DNA was higher than that of *Act1* DNA for all RNA samples from glucose-starved cells, and vice versa for all RNA samples from glucose-provided cells.

[0054] These results are consistent with the previous blot analysis of RNA from intact suspension cells (Sheu et al., J. Biol. Chem., 271:26998-27004, 1996). When the amounts of PCR products as shown in ethidium bromide-stained agarose gel were quantified and plotted against the amounts of input RNA used for CRT-PCR, a linear relationship was  
50 observed between the levels of the CRT-PCR products and the concentrations of input mRNAs. Thus, the relative amount of mRNA can be estimated from its CRT-PCR product.

### (2) Effect of the $\alpha$ Amy3 3'UTR on mRNA Accumulation as Determined by CRT-PCR Analyses

55 [0055] To determine whether the 3'UTR of  $\alpha$ *Amy3* functions as a determinant of mRNA stability in response to sugar availability, a series of constructs (Fig. 1) were prepared. Plasmids pLAm containing *Ubi-Luc- $\alpha$ Amy3*, pLAc containing *Ubi-Luc-Act1*, and pLN containing *Ubi-Luc-Nos* were transfected into rice protoplasts. The protoplasts were then incubated in a glucose-containing or glucose-free medium.

[0056] Glucose, instead of sucrose, was used as the carbon source here. The reason was that rice protoplasts contain no cell walls and hence no cell wall-bound invertase, which hydrolyzes sucrose into glucose and fructose before up-take by intact suspension cells (Yu et al., *J. Biol. Chem.*, 266:21131-21137, 1991), and that sucrose cannot be transported efficiently into protoplasts. It has been shown that glucose represses  $\alpha$ -amylase gene expression as well as sucrose does (Yu et al., *J. Biol. Chem.*, 266:21131-21137, 1991).

[0057] Total RNA was purified from the protoplasts, and CRT-PCR was performed using multiple sets of primers which simultaneously amplified cDNAs derived from the endogenous *Act1* and  $\alpha$ *Amy3* mRNAs, and from the foreign chimeric *Luc*- $\alpha$ *Amy3*, *Luc*-*Act1*, or *Luc*-*Nos* mRNAs (Fig. 1).

[0058] The data showed that, in both transfected and non-transfected protoplasts, accumulation of the endogenous *Act1* mRNA was enhanced by glucose, and that of the  $\alpha$ *Amy3* mRNA was suppressed by glucose. This result indicates that expression of the endogenous *Act1* and  $\alpha$ *Amy3* is subject to opposite regulation by glucose in protoplasts as well as in intact suspension cells (Sheu et al., *J. Biol. Chem.* 271:26998-27004, 1996). Accumulation of the *Luc*- $\alpha$ *Amy3* mRNA in pLAm-transfected protoplasts was also suppressed by glucose. In contrast, accumulation of the *Luc*-*Act1* mRNA in pLAc-transfected protoplasts, and accumulation of the *Luc*-*Nos* mRNA in pLN-transfected protoplasts, were both enhanced by glucose. These results demonstrate that the  $\alpha$ *Amy3* 3'UTR enhances, while the *Act1* and *Nos* 3'UTRs reduce, accumulation of the *Luc* mRNA in glucose-starved cells.

[0059] In the above experiments, the expression of endogenous *Act1* and  $\alpha$ *Amy3* was used as an internal control for indication of normal physiology in response to availability of glucose.

[0060] A reference gene can also be co-transfected with the test genes, with its expression used as an internal control. For instance, plasmid pGN containing *Ubi-Gus-Nos* was co-transfected with pLN or pLAm into rice protoplasts which were subsequently incubated in a medium with or without glucose. CRT-PCR was performed using primers that amplified cDNAs derived from the foreign chimeric *Gus-Nos*, *Luc-Nos*, or *Luc*- $\alpha$ *Amy3* mRNAs (Fig. 1). The data showed that accumulation of the *Gus-Nos* mRNA, like that of the *Luc-Nos* mRNAs, was enhanced by glucose. In contrast, accumulation of the *Luc*- $\alpha$ *Amy3* mRNA was repressed by glucose. This result further demonstrates that the  $\alpha$ *Amy3* 3'UTR specifically enhances the accumulation of reporter mRNA in response to glucose starvation.

### (3) Effect of the $\alpha$ *Amy3* 3'UTR on mRNA Accumulation as Determined by Primer Extension Analysis

[0061] The effect of the  $\alpha$ *Amy3* 3'UTR on mRNA accumulation was confirmed with another approach - primer extension analysis. Plasmids pLAm and pLAc were transfected into rice protoplasts which were then incubated in a medium with or without glucose. Total RNA was purified from the protoplasts and used as templates for primer extension analysis using primer 3RT2 or A30 (Table 1) that was also used in CRT-PCR analyses (Fig. 1). Levels of the extension products derived from the endogenous  $\alpha$ *Amy3* (1.7 kb) and the chimeric *Luc*- $\alpha$ *Amy3* (2.1 kb) mRNAs in pLAm-transfected protoplasts were shown to be reduced by glucose. In contrast, levels of the extension products derived from the endogenous *Act1* (1.4 kb) and the chimeric *Luc*-*Act1* (2.1 kb) mRNAs in pLAc-transfected protoplasts were enhanced by glucose.

[0062] This result was consistent with that obtained with CRT-PCR, confirming that the  $\alpha$ *Amy3* 3'UTR plays a role in the sugar-dependent repression of mRNA accumulation.

### (4) Kinetics of Accumulation of mRNA Carrying the $\alpha$ *Amy3* 3'UTR in Protoplasts

[0063] To analyze the kinetics of accumulation of a reporter mRNA carrying the  $\alpha$ *Amy3* 3'UTR, rice protoplasts were transfected with pLAm and then incubated for various lengths of time in a medium with or without glucose. CRT-PCR analysis showed that, within 2 days, levels of *Act1* mRNA remained high in the presence of glucose, but decreased with time in the absence of glucose. In contrast, levels of both the endogenous  $\alpha$ *Amy3* and chimeric *Luc*- $\alpha$ *Amy3* mRNAs were initially low in the presence of glucose, but increased significantly within 12 hr following glucose depletion. At day 2, levels of *Act1* and  $\alpha$ *Amy3* mRNAs in transfected protoplasts in the presence or absence of glucose, respectively, were similar to those in non-transfected protoplasts. This result suggests that the sugar-dependent expression pattern of the foreign chimeric gene is similar to that of the endogenous gene.

[0064] Similar experiments were performed to define more precisely the time required for induction of *Luc*- $\alpha$ *Amy3* mRNA accumulation by glucose depletion. Levels of both endogenous  $\alpha$ *Amy3* and chimeric *Luc*- $\alpha$ *Amy3* mRNAs were found to increase significantly within 2 h after depletion of glucose, confirming that accumulation of the *Luc* mRNA carrying the  $\alpha$ *Amy3* 3'UTR has the same kinetics as that of the endogenous  $\alpha$ *Amy3* mRNA.

### (5) 5' Deletion Analysis of the $\alpha$ *Amy3* 3'UTR

[0065] To identify sequences in the  $\alpha$ *Amy3* 3'UTR that determine the sugar-dependent instability of mRNA, a series of 5' deletions of the  $\alpha$ *Amy3* 3'UTR were generated. The truncated  $\alpha$ *Amy3* 3'UTRs were inserted downstream of the

*Ubi* promoter-*Luc* coding region (Fig. 4). The resulting constructs were then transfected into rice protoplasts and their expression analyzed by CRT-PCR. CRT-PCR products were analyzed on agarose gel.

[0066] Accumulations of the endogenous *Act1* mRNA were shown to be enhanced by glucose, and those of the  $\alpha$ *Amy3* mRNA were suppressed by glucose, in either transfected or non-transfected protoplasts. Accumulations of the wild type or mutant *Luc*- $\alpha$ *Amy3* mRNA in protoplasts transfected with pLAm, pLAmD1, pLAmD2, pLAmD5, and pLAmD6 were suppressed by glucose. In contrast, accumulations of the mutant *Luc*- $\alpha$ *Amy3* mRNA in protoplasts transfected with pLAmD3, pLAmD4, and pLAmD7 were enhanced by glucose. This result indicates that multiple regions in the  $\alpha$ *Amy3* 3'UTR repress or de-repress the accumulation of *Luc* mRNA in a sugar-dependent manner.

[0067] To use a reference gene as an internal control, pGN was cotransfected with each of the wild type or mutant plasmids used in the experiments into the rice protoplasts. Accumulation of mRNAs determined by CRT-PCR was as expected.

[0068] To test whether the effect of deletion on mRNA accumulation changed with different coding sequences, three DNA fragments containing the deleted  $\alpha$ *Amy3* 3'UTRs were chosen and fused downstream of the *Ubi* promoter-*Gus* coding region (Fig. 4). The resulting constructs were then cotransfected with pLN into rice protoplasts and their expression analyzed by CRT-PCR. Levels of the *Luc*-*Nos* mRNA were shown to be higher in the presence of glucose than in the absence of glucose. This result was consistent with the results in Part (2). Accumulations of the wild type or mutant *Gus*- $\alpha$ *Amy3* mRNA in protoplasts transfected with pGAm, pGAmD2, and pGAmD5 were suppressed by glucose. In contrast, accumulation of the mutant *Gus*- $\alpha$ *Amy3* mRNA in protoplasts transfected with pGAmD3 was enhanced by glucose. These results demonstrate that the sugar-dependent repression or derepression of mRNA accumulation controlled by multiple sequences in the  $\alpha$ *Amy3* 3'UTR is independent of the connected coding sequences.

#### (6) Functional Analysis of the Multiple Sugar-Responsive Regions in the $\alpha$ *Amy3* 3'UTR

[0069] As shown by the above deletional analysis, in the absence of glucose, the sequences in the  $\alpha$ *Amy3* 3'UTR spanning nucleotides 1 to 124 and 184 to 258 were involved in enhancing mRNA accumulation, whereas the sequence spanning nucleotides 124 to 184 were involved in suppressing mRNA accumulation.

[0070] To investigate whether these regions by themselves can play the same roles, DNA fragments containing the  $\alpha$ *Amy3* 3'UTR from sequences 3 to 135 (fragment I), 119 to 199 (fragment II), and 182 to 276 (fragment III) were amplified by PCR. These fragments were inserted between the *Ubi* promoter-*Luc* coding region and the *Nos* 3'UTR (Fig. 1) to generate pLIN, pLIIN, and pLIIN, respectively (see also Example 1, *supra*). The resulting constructs were then cotransfected with pGN into rice protoplasts and their expression analyzed by CRT-PCR.

[0071] The CRT-PCR analysis showed that the *Gus*-*Nos* mRNA levels (internal control) were enhanced by glucose as expected, and the accumulation of the *Luc*-I-*Nos* or *Luc*-III-*Nos* mRNA in protoplasts containing pLIN or pLIIN was suppressed by glucose. On the contrary, accumulation of the *Luc*-II-*Nos* mRNA in protoplasts transfected with pLIIN was enhanced by glucose.

[0072] Thus, the above analyses demonstrate that at least two regions (or subdomains) in the  $\alpha$ *Amy3* 3'UTR could confer a sugar-dependent repression of accumulation of the *Luc*-*Nos* mRNA whose accumulation otherwise was enhanced by sugar. These results were consistent with those obtained by RNA dot blot analysis (see Example 1, *supra*, e.g., Fig. 21).

#### Other Embodiments

[0073] It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

[0074] Other aspects, advantages, and modifications are within the scope of the following claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

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Chan, Ming-Tsair

(ii) TITLE OF THE INVENTION: 3' UNTRANSLATED REGIONS OF  
ALPHA-AMYLASE GENES

(iii) NUMBER OF SEQUENCES: 17

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(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: Windows95  
(D) SOFTWARE: FastSEQ for Windows Version 2.0

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
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(C) CLASSIFICATION:

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(B) FILING DATE:

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(A) TELEPHONE: 617-542-5070  
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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 320 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGGCTCAAG	CCCTAAACTG	AACGGGATAG	TCATGCTCAA	ACCAGTTTCT	ACACGGCAAG	60
AATTTACTGA	TTCTTATACT	TTTGCACTCA	ATTAAATTAT	GGTTTTTATA	TATGTAATTT	120
TGTATCCGAT	TGTAGCGTTC	GAATAAGTAG	GCAGGCTCTC	TAGCCTCTAG	GTTAATTGCG	180
GGGCATATGT	AGCTTGCCAG	TTAATTGTGT	TTGTATCACG	CAGTTTGTA	CCGTTGGTGC	240
ATATATATGT	CAGGTTTCAGG	TTCAGGATGC	AGTAAAAAAT	CATACTGCAC	CGATCAGTGA	300
GTTTTTATAT	ACTGGTTTTA					320

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TAATCTAGAT AGCGGGCTCA AGCCCTA

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCCCCGCGGT CAGTGAGTTT TTA

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATATAATGT CAGGTT

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGCTCTAGAT AATTCTTCGA CCCA

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCCCCGCGGT GGTACCCGCA TCA

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGGGACTCT SSTCATAAAA

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACGGCATCAA CGCCGGGAGC

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGATGGACA GGTTCACC

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCAGCGATGA CGAAATTCTT

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCTCTTTGAT GTGCTGTGCC

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGAGCTCGGC TCAAGCCCTA AA

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGAGCTCCTA CAATCGGATA CA

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGAGCTCTTG TATCCGATTG TA

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TGAGCTCTGG CAAGCTACAT AT

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGAGCTCGGC ATATGTAGCT T

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGAGCTCCCT GAACCTGAAC CT

22

## Claims

1. An expression vector comprising a promoter capable of directing expression of a coding sequence in an angiosperm cell, and a 3' untranslated region of a rice  $\alpha$ -amylase gene, wherein, after the coding sequence is inserted into the vector, the 3' untranslated region and the coding sequence are transcribed into a single mRNA.
2. The vector of claim 1, wherein said  $\alpha$ -amylase gene is an  $\alpha$ Amy3 gene.
3. The vector of claim 2, wherein said 3' untranslated region consists of 20 or more consecutive nucleotides from SEQ ID NO:1.
4. The vector of claim 1, further comprising a signal sequence encoding a signal peptide, wherein said signal sequence is situated downstream of said promoter.
5. The vector of claim 4, wherein said signal sequence is derived from an  $\alpha$ -amylase gene.
6. The vector of claim 5, wherein said signal sequence is derived from a rice  $\alpha$ Amy8 gene.
7. The vector of claim 4, wherein said  $\alpha$ -amylase gene is an  $\alpha$ Amy3 gene.
8. The vector of claim 7, wherein said 3' untranslated region consists of 20 or more consecutive nucleotides from SEQ ID NO:1.
9. A method of producing a polypeptide in an angiosperm cell, said method comprising:
  - providing an expression construct which comprises (i) a coding sequence for the polypeptide, (ii) a promoter capable of directing expression of the coding sequence in the angiosperm cell, and (iii) a 3' untranslated region of a rice  $\alpha$ -amylase gene, wherein the coding sequence and the untranslated region, which are not naturally transcribed into a single mRNA, are positioned for transcription into a single mRNA;
  - transforming an angiosperm host cell with the expression construct; and
  - subjecting the transformant host cell to a sugar-free environment to promote expression of the polypeptide.
10. The method of claim 9, wherein said  $\alpha$ -amylase gene is an  $\alpha$ Amy3 gene.
11. The method of claim 10, wherein the 3' untranslated region consists of 20 or more consecutive nucleotides of SEQ ID NO:1.
12. The method of claim 11, wherein the expression construct further comprises, between the promoter and the coding sequence, a signal sequence encoding a signal peptide.
13. The method of claim 12, wherein the signal sequence is derived from a rice  $\alpha$ Amy8 gene.



14. A method of producing a polypeptide in an angiosperm cell, said method comprising:

providing an expression construct which comprises (i) a coding sequence for the polypeptide, (ii) a promoter capable of directing expression of the coding sequence in the angiosperm cell, and (iii) a 3' untranslated region of a rice  $\alpha$ -amylase gene, wherein the coding sequence and the untranslated region, which are not naturally transcribed into a single mRNA, are positioned for transcription into a single mRNA;  
transforming an angiosperm host cell with the construct; and  
subjecting the transformant host cell to a sugar-containing environment to promote expression of the polypeptide.

15. The method of claim 14, wherein said  $\alpha$ -amylase gene is an  $\alpha$ Amy3 gene.

16. The method of claim 15, wherein the 3' untranslated region consists of 20 or more consecutive nucleotides of SEQ ID NO:1.

17. The method of claim 16, wherein the expression construct further comprises, between the promoter and the coding sequence, a signal sequence encoding a signal peptide.

18. The method of claim 17, wherein the signal sequence is derived from a rice  $\alpha$ Amy8 gene.

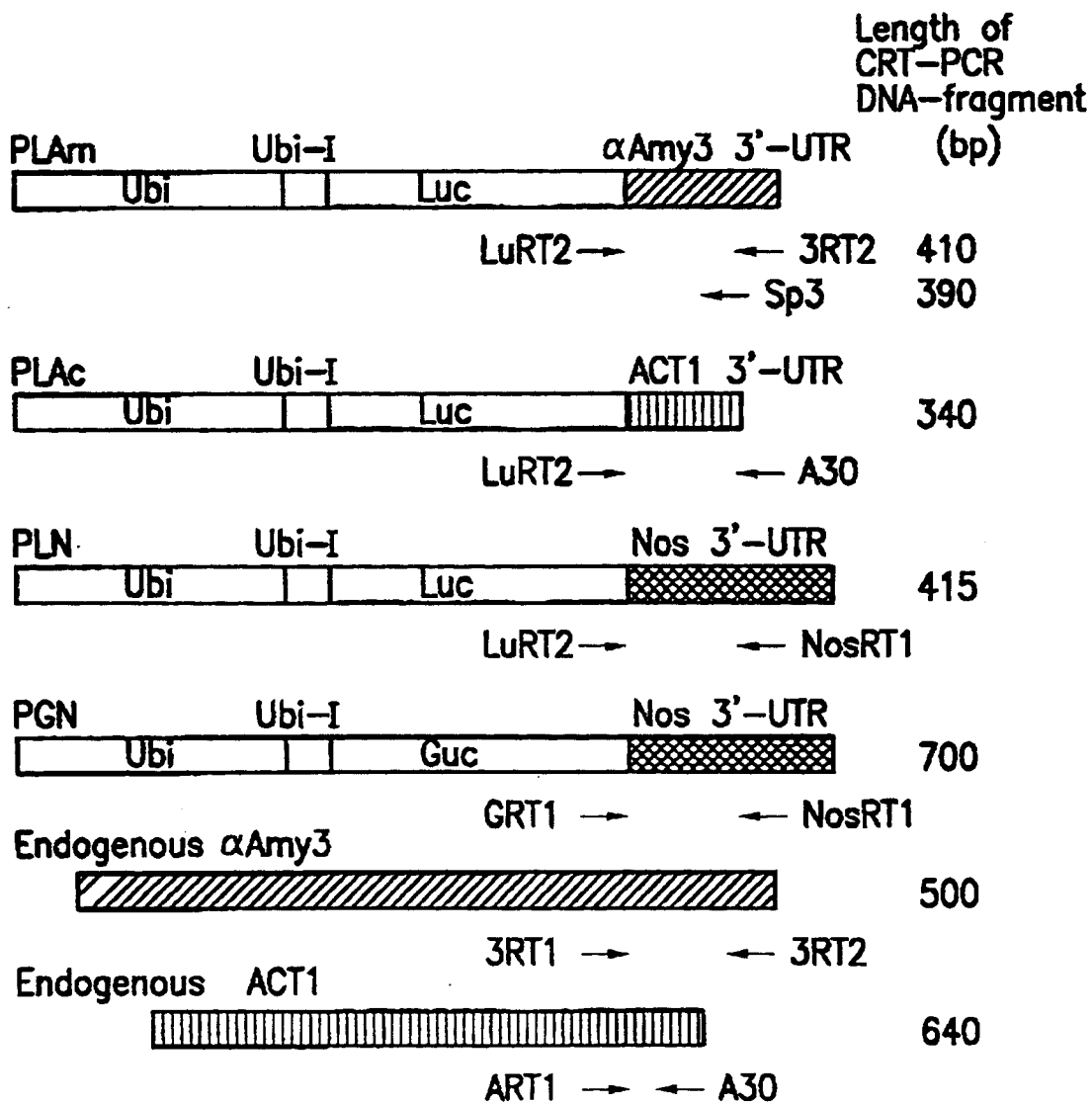
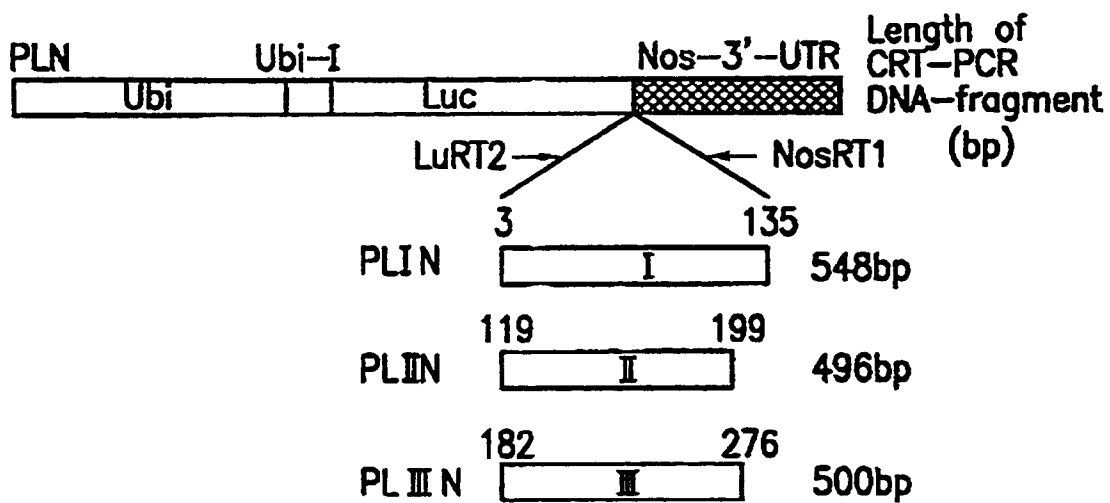


FIG. 1

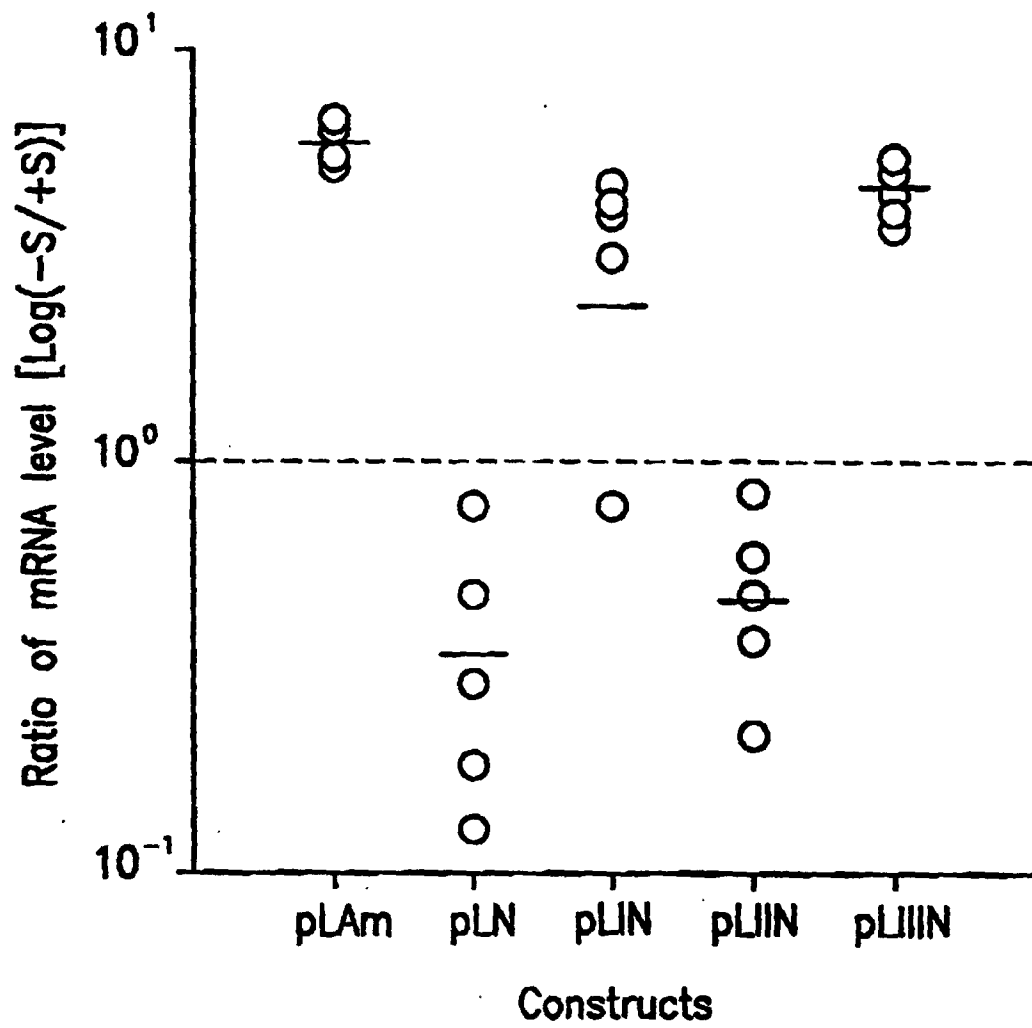


FIG. 2

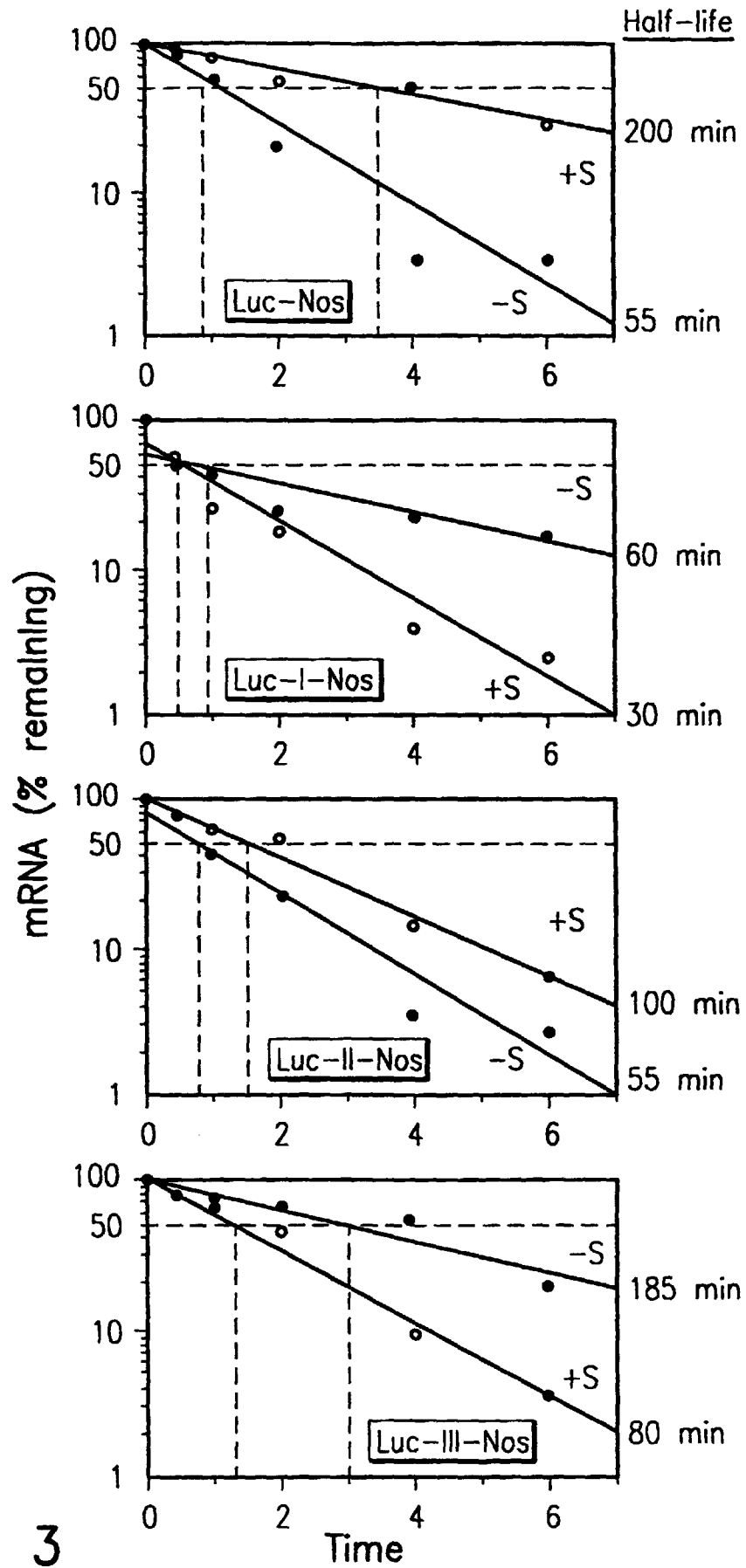


FIG. 3



FIG. 4